THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1989 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 264, No. 28, Issue of October 5, pp. 16700-16712, 1989
Printed in U.S.A.

Universality and Structure of the N-end Rule*

(Received for publication, February 2, 1989)

David K. Gonda‡§, Andreas Bachmair‡¶, Ingrid Wünning‡‖, John W. Tobias‡, William S. Lane‡‡, and Alexander Varshavsky‡**

From the ‡Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 and the ‡‡Harvard Biological Laboratories, Harvard Microchemistry Facility, Harvard University, Cambridge, Massachusetts 02138

Our previous work has shown that, in the yeast *Saccharomyces cerevisiae*, any of the eight *stabilizing* amino-terminal residues confers a long (>20 h) half-life on a test protein β-galactosidase (βgal), whereas 12 *destabilizing* amino-terminal residues confer on βgal half-lives from less than 3 min to 30 min. We now show that an analogous single-residue code (the N-end rule) operates in an *in vitro* system derived from mammalian reticulocytes. We also show that the N-end rule has a hierarchical structure. Specifically, amino-terminal Glu and Asp (and also Cys in reticulocytes) are *secondary* destabilizing residues in that they are destabilizing through their ability to be conjugated to *primary* destabilizing residues such as Arg. Amino-terminal Gln and Asn are *tertiary* destabilizing residues in that they are destabilizing through their ability to be converted, via selective deamidation, into secondary destabilizing residues Glu and Asp. Furthermore, in reticulocytes, distinct types of the N-end-recognizing activity are shown to be specific for three classes of primary destabilizing residues: basic (Arg, Lys, His), bulky hydrophobic (Phe, Leu, Trp, Tyr), and small uncharged (Ala, Ser, Thr). Features of the N-end rule in reticulocytes suggest that the exact form of the N-end rule may depend on the cell's physiological state, thereby providing a mechanism for selective destruction of preexisting proteins upon cell differentiation.

The half-lives of intracellular proteins range from a few seconds to many days. Rates of selective protein degradation are a function of the cell's physiological state and appear to be controlled differentially for individual proteins. In particular, most of the damaged and otherwise abnormal proteins are metabolically unstable (reviewed by Finley and Varshavsky, 1985; Hershko and Ciechanover, 1986; Bond and Butler, 1987; Dice, 1987; Hershko, 1988; Arfin and Bradshaw, 1988). Many undamaged proteins are also short-lived *in vivo* (Gottesman *et al.*, 1981; Spindler and Berk, 1984; Cheng and Echols, 1987). Metabolic instability of these proteins allows for efficient temporal control of their intracellular concentrations and has been shown to be essential for the regulatory functions of several eukaryotic and bacterial proteins (Nasmyth, 1983; Ho *et al.*, 1986; Banuett *et al.*, 1986; Straus *et al.*, 1987; Bahl *et al.*, 1987; Shanklin *et al.*, 1987). Many other proteins, while long-lived as components of larger macromolecular complexes such as ribosomes and oligomeric proteins, are metabolically unstable in a free, unassociated state (Maicas *et al.*, 1988; Tsay *et al.*, 1988).

Most of the selective degradation of intracellular proteins under normal metabolic conditions is adenosine triphosphate (ATP)-dependent and (in eukaryotes) nonlysosomal. Both biochemical and genetic evidence indicates that, in eukaryotes, covalent conjugation of ubiquitin, a 76-residue protein, to short-lived intracellular proteins is essential for their selective degradation (Hershko *et al.*, 1980; Finley *et al.*, 1984; Ciechanover *et al.*, 1984). This understanding, however, leaves unsolved the problem of targeting: how are intracellular proteins initially recognized as proteolytic substrates? Using a new approach, which makes it possible to expose *in vivo* different amino acid residues at the amino termini of otherwise identical test proteins, we have found that, in the yeast *S. cerevisiae*, an important component of a protein's degradation signal is remarkably simple and consists of the protein's amino-terminal residue (Bachmair *et al.*, 1986). The resulting code or rule that relates the protein's metabolic stability and the nature of its amino-terminal residue has been called the N-end rule (Bachmair *et al.*, 1986).

In the present work, we show that an N-end rule similar, but not identical to the yeast N-end rule can be defined in mammalian reticulocytes. Certain features of the reticulocyte N-end rule suggest that an N-end rule-mediated degradative pathway may underlie selective destruction of preexisting, otherwise long-lived proteins upon cell differentiation. We also show that the N-end rule is organized hierarchically, and analyze mechanistic aspects of its organization.

EXPERIMENTAL PROCEDURES

Expression Vectors Encoding Ub-X-βgal Proteins—Four of the pKKUb-*X*-βgal vectors (those encoding Ub-Met-βgal, Ub-Gln-βgal, Ub-Arg-βgal, and Ub-Pro-βgal) were constructed as follows. Oligonucleotide-directed mutagenesis (Smith, 1985; Ausubel *et al.*, 1987) was used to insert the sequence GTAC between the first and second codons of the ubiquitin reading frame in the yeast expression vector pUB23 (which encodes Ub-Met-βgal) and in its derivatives (Bachmair *et al.*, 1986) encoding Ub-Arg-βgal, Ub-Gln-βgal, and Ub-Pro-βgal. The insertion created a *Kpn*I site positioned such that when the vector is cut with *Kpn*I and the ends blunted by mung bean nuclease, the second codon of the ubiquitin reading frame starts precisely at one of the fragment's ends. Thus, digestion of each of the above four vectors with *Kpn*I and *Tth*111I, followed by treatment with mung bean nuclease, yielded four fragments which contained the corre-

* This work was supported in part by Grants GM31530 and DK39520 (to A. V.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

¶ Fellow of the European Molecular Biology Organization. Present address: Max-Planck-Institut für Züchtungsforschung, Abteilung Genetische Grundlagen der Pflanzenzüchtung, Egelspfad, D-5000 Köln 30, Federal Republic of Germany.

‖ Fellow of Deutsche Forschungsgemeinschaft. Present address: Dept. of Microbiology, State University of New York, Stony Brook, NY 11794.

**To whom correspondence should be addressed: Dept. of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

sponding Ub-*X*-βgal-coding sequences but lacked the first (ATG) codon of the ubiquitin reading frame. These fragments were subcloned into an *Escherichia coli* expression vector pKK233-2 (Amann and Brosius, 1985) which had been prepared by digesting with *Nco*I and filling in staggered ends using Klenow fragment of *Pol*I (Ausubel *et al.*, 1987). This step yielded the complete Ub-*X*-βgal sequence (in which the ATG codon was supplied by the pKK233-2 vector), optimally positioned downstream of the P*trc* promoter of the vector. To construct the remaining 16 pKKUb-*X*-βgal expression vectors, pKKUb-Arg-βgal was digested with *Sal*I and *Bam*HI. One of the two *Bam*HI sites in pKKUb-Arg-βgal is located at the junction between the ubiquitin- and βgal-coding sequences; the other *Bam*HI site, present in the initial pKK233-2 vector (Amann and Brosius, 1985), was removed in a preliminary construction step. The small *Sal*I/*Bam*HI fragment (containing the P*trc* promoter, the complete ubiquitin-coding sequence, and the Arg codon at the Ub-βgal junction) was subcloned into a M13mp9 vector (Messing and Vieira, 1982; Ausubel *et al.*, 1987). A *Bst*XI/*Bam*HI fragment of this construct that contained a portion of the ubiquitin-coding sequence and the Arg codon at the Ub-βgal junction, was then exchanged for the 16 otherwise identical *Bst*XI/*Bam*HI fragments (from the previously made, M13mp9-based constructs (Bachmair *et al.*, 1986)) which differed exclusively in a codon at the Ub-βgal junction. The resulting 16 M13mp9-based constructs were treated with *Sal*I and *Bam*HI, and the small *Sal*I/*Bam*HI fragments containing the ubiquitin-coding sequence and different single codons at the Ub-βgal junction were cloned back into pKKUb-Arg-βgal, replacing the original *Sal*I/*Bam*HI fragment, and yielding the remaining sixteen pKKUb-*X*-βgal expression vectors. In all cases, the identity of the amino acid encoded at the Ub-βgal junction of a final pKKUb-*X*-βgal construct was verified by subcloning into M13 and nucleotide sequencing by the chain termination method (Ausubel *et al.*, 1987).

Production of X-βgal by Cleavage with Factor Xa Protease—The small *Sal*I/*Bam*HI fragment of pKKUb-Phe-βgal (see above) was subcloned into M13mp19 (Messing and Vieira, 1982; Ausubel *et al.*, 1987) where the last four codons of the ubiquitin reading frame were changed, using oligonucleotide-directed mutagenesis, into those encoding the recognition site (-Ile-Glu-Gly-Arg-) for Factor Xa protease (Nagai and Thogersen, 1987). The mutagenesis protocol was that specified by the Bio-Rad Mutagene Kit (Bio-Rad). The desired mutants were identified by sequencing of DNA (Ausubel *et al.*, 1987) from individual phage isolates. The construct obtained was subjected to a second round of oligonucleotide-directed mutagenesis to change Phe, which abuts the carboxyl-terminal end of the Factor Xa cleavage site, into Gln. The resulting M13mp19-based construct and the pKKUb-Phe-βgal vector (see above) were both cut with *Sal*I and *Bam*HI, and the small *Sal*I/*Bam*HI fragment of the M13mp19-based construct was ligated to the large *Sal*I/*Bam*HI fragment of pKKUb-Phe-βgal, yielding the expression vector pKKUb-(Xa)-Gln-βgal. The corresponding protein, Ub-(Xa)-Gln-βgal (in which the last 4 residues of ubiquitin were changed as described above) was overexpressed in *E. coli* (strain MV1190) and purified by affinity chromatography (see below). The purified Ub-(Xa)-Gln-βgal (0.1–0.3 mg/ml) was incubated for 24 h at room temperature in 12% glycerol, 0.35 M NaCl, 0.25 mM $MgCl_2$, 0.11 M Tris-HCl, pH 8.3, containing 1 mg/ml bovine serum albumin and 5 units/ml of Factor Xa from human plasma (Boehringer Mannheim). Cleavage of Ub-(Xa)-Gln-βgal with Factor Xa was verified by SDS-PAGE[1] and by amino acid sequencing (see below). Otherwise identical digestions of Ub-Gln-βgal that lacked the Factor Xa cleavage site did not yield proteolytic fragments.

Purification of Ub-X-βgal Proteins Expressed in E. coli—An overnight culture (1 ml) of *E. coli* JM101 cells bearing one of the 20 pKKUb-*X*-βgal expression vectors (see above) was diluted into 50 ml of Luria broth supplemented with ampicillin at 40 μg/ml, and the cells were grown with shaking for approximately 2 h at 37 °C. The cells were harvested by centrifugation at 4,000 × *g* for 10 min, washed twice with M9 buffer, and resuspended in 25 ml of M9 minimal medium supplemented with glucose (0.22%, w/v), thiamine (18 μg/ml), ampicillin (40 μg/ml), 0.5 mM isopropylthiogalactoside, and 0.15 ml of 10.5% (w/v) Methionine Assay Medium (Difco). After incubation with shaking for 1 h at 37 °C, 0.5–1.0 mCi of Trans-^{35}S-label (ICN; ~85% [^{35}S]methionine, ~15% [^{35}S]cysteine) was added and shaking was continued for 5 min. Unlabeled L-methionine was then added to 1 mM and shaking was continued for another 10 min. Cells were harvested, washed twice with M9 buffer, and resuspended in 0.5 ml of 25% (w/v) sucrose, 50 mM Tris-HCl, pH 8.0. Thereafter, 0.1 ml of lysozyme (10 mg/ml; Sigma) in 0.25 M Tris-HCl, pH 8.0, was added, and the mixture was incubated at 0 °C for 5 min, followed by the addition of 0.1 ml of 0.5 M Na-EDTA, pH 8.0, and further incubation at 0 °C for 5 min. The cell suspension was then added to a lysis solution (0.8 ml H_2O, 50 μl of 1 M Tris-HCl, pH 8.0, 125 μl of 0.5 M Na-EDTA, pH 8.0, 10 μl of 10% (v/v) Triton X-100), and gently mixed. The lysate was centrifuged at 40,000 × *g* for 1 h, and Ub-*X*-βgal was purified from the supernatant by affinity chromatography on aminophenylthiopyranogalactoside-Sepharose (APTG-Sepharose) as described by Ullman (1984). Ubiquitin-*X*-βgal was eluted from APTG-agarose with 10 mM 2-mercaptoethanol, 0.1 M Na-borate, pH 10.0, dialyzed overnight at 4 °C against 50% (v/v) glycerol, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM $MgCl_2$, 40 mM Tris-HCl, pH 7.5, and stored at −20 °C in the same buffer. Control experiments showed that the transient exposure of Ub-*X*-βgal to the high pH elution buffer does not result in a detectable change of βgal enzymatic activity. Typical yields of [^{35}S]Ub-*X*-βgal purified by the above procedure were 0.5–1 mg, with enzymatic activity of 4–6 × 10^4 units/mg and specific radioactivity of 1–2 × 10^5 cpm/μg. Unlabeled Ub-*X*-βgal was prepared essentially as described above except that after 2 h of growth in Luria broth with ampicillin, isopropylthiogalactoside was added to 0.5 mM, and the cells were grown for one more hour before harvesting and lysis.

In Vitro Assays with Reticulocyte Extract—Washed reticulocytes from phenylhydrazine-treated rabbits were purchased from Green Hectares (Oregon, WI) and shipped overnight at 0 °C. The reticulocytes were washed three times with 3–4 volumes of standard phosphate-buffered saline (centrifugations at 1,000 × *g* for 10 min at 4 °C). To deplete intracellular ATP (Hershko *et al.*, 1980), the cells were incubated for 90 min at 37 °C in Krebs-Ringer phosphate buffer containing 0.2 mM 2,4-dinitrophenol and 20 mM 2-deoxyglucose, and then washed three times in phosphate-buffered saline. Pelleted reticulocytes were then lysed at 0 °C by resuspending the pellet in 1.5 volumes of 1 mM DTT. After ~10 min at 0 °C, the sample was centrifuged at 80,000 × *g* for 90 min at 4 °C. The supernatant was removed, divided into aliquots, and stored under liquid nitrogen. Only once-frozen aliquots were used in all experiments. Unless stated otherwise, the ATP-depleted reticulocyte extract was used directly after thawing, without further processing. In some experiments, the thawed extract was first dialyzed overnight at 4 °C against 1 mM DTT, 10 mM Tris-HCl, pH 7.5, in dialysis tubing with a M_r cutoff of ~3 kDa. Fraction II was prepared by DEAE chromatography of ATP-depleted reticulocyte extract as previously described (Hershko *et al.*, 1980) and stored under liquid nitrogen. Reaction mixtures for assaying the degradation of test proteins in either the total reticulocyte extract or Fraction II contained (final concentrations) 5% (v/v) glycerol, 1 mM DTT, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5, 70% (v/v) reticulocyte extract (or Fraction II at 6 mg/ml of the total protein), [^{35}S]Ub-*X*-βgal fusion protein at 20 μg/ml, and, when present, 0.5 mM ATP and an ATP-regenerating system (10 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase). Reaction mixtures were prepared as follows: a mixture complete except for ATP and ATP-regenerating system was incubated for 10 min at 37 °C to allow for the deubiquitination of a Ub-*X*-βgal fusion protein (see Fig. 2, *lanes d–o*); ATP and ATP-regenerating system were then added to start the ATP-dependent reactions in the extract (time zero in Figs. 3 and 4), and the 37 °C incubation continued. Reaction mixtures containing Fraction II were also supplemented with purified ubiquitin at 0.1 mg/ml. Control reactions with the ATP-depleted extract were performed identically except that ATP and ATP-regenerating system were omitted. The ATP-dependent degradation of ^{125}I-labeled bovine serum albumin, hen lysozyme, and cytochrome *c* from *S. cerevisiae* (purchased from Sigma, and labeled using the chloramine-T method (Hershko *et al.*, 1980)) was assayed as described above except that the 10-min preincubation of the test protein at 37 °C in the ATP-depleted reticulocyte extract was omitted. To follow the degradation of test proteins, aliquots were taken from the reaction mixture at the indicated times, and either assayed for the amount of 5% trichloroacetic acid-soluble radioactivity present, or analyzed by SDS-PAGE (Laemmli, 1970; Ausubel *et al.*, 1987) (8% polyacrylamide, 0.05% bisacrylamide, 15 × 15 × 0.15 cm gels), with subsequent fluorography.

Amino Acid Sequencing—For the amino acid sequencing by automated Edman degradation, *X*-βgal test proteins were reisolated by immunoprecipitation with a monoclonal antibody to βgal (Bachmair *et al.*, 1986) from either reticulocyte extract, reticulocyte Fraction II (see above), or extracts made from βgal-containing yeast cells (Bach-

[1]The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; APTG, aminophenylthiopyranogalactoside.

mair *et al.*, 1986; Bachmair and Varshavsky, 1989). The immunoprecipitated *X*-βgal proteins were subjected to SDS-PAGE and then electroblotted onto polyvinylidene difluoride membrane (Matsudaira, 1987). Amino acid sequencing of 10–30 pmol of electroblotted protein was carried out for a minimum of 5 cycles using an Applied Biosystems 470A Protein Sequencer equipped with an online 120A PTH Analyzer.

RESULTS

Expression and Purification of Ub-βgal Fusion Proteins—To produce otherwise identical β-galactosidase (βgal) test proteins bearing different amino-terminal residues, Bachmair *et al.* (1986) used a construct that expresses a ubiquitin-βgal fusion protein in yeast. The nascent Ub-βgal is rapidly and precisely deubiquitinated *in vivo* by an endogenous processing protease to yield a βgal test protein bearing the desired amino-terminal residue (Fig. 1, *A* and *B*, and Table I; see also Bachmair *et al.*, 1986; Bachmair and Varshavsky, 1989).

In the present work, the ubiquitin fusion approach (Fig. 1*C*) was used to analyze the N-end rule in an extensively characterized *in vitro* ATP-dependent proteolysis system (Etlinger and Goldberg, 1977; Hershko *et al.*, 1980; Hershko, 1988) derived from rabbit reticulocytes. We have constructed a set of *E. coli* expression vectors encoding Ub-*X*-βgal fusion proteins which differ exclusively at residue *X* at the Ub-βgal junction (see "Experimental Procedures"). Metabolically labeled [^{35}S]Ub-*X*-βgal proteins were purified from *E. coli* using affinity chromatography (Fig. 2, *lanes a–c*). None of the Ub-*X*-βgal proteins were significantly deubiquitinated or otherwise degraded in *E. coli*, apparently because bacteria lack the eukaryotic ubiquitin system (Bachmair *et al.*, 1986; Finley *et al.*, 1988).

Deubiquitination of Ub-X-βgal Fusion Proteins in ATP-depleted Reticulocyte Extract—Each of the 20 ^{35}S-labeled Ub-*X*-βgal proteins was added to an extract prepared from ATP-depleted rabbit reticulocytes (see "Experimental Procedures"), and the fates of the added proteins were followed by SDS-PAGE (Fig. 2, *lanes d–s*). As has been previously observed with the same ubiquitin fusions in yeast cells (Bachmair *et al.*, 1986), an apparently ubiquitin-specific protease in reticulocyte extract deubiquitinated the added Ub-*X*-βgal fusion proteins to yield the corresponding *X*-βgal test proteins. The deubiquitination of 19 of the 20 Ub-*X*-βgal proteins in the ATP-depleted extract was more than 90% complete in 5 min at 37 °C (Fig. 2, *lanes d–o*, Table I, and data not shown). The single exception, both in yeast (Bachmair *et al.*, 1986) and in reticulocytes, is Ub-Pro-βgal, which was deubiquitinated approximately 20 times more slowly than were the other Ub-*X*-βgal proteins (Fig. 2, *lanes p–s*, and data not shown).

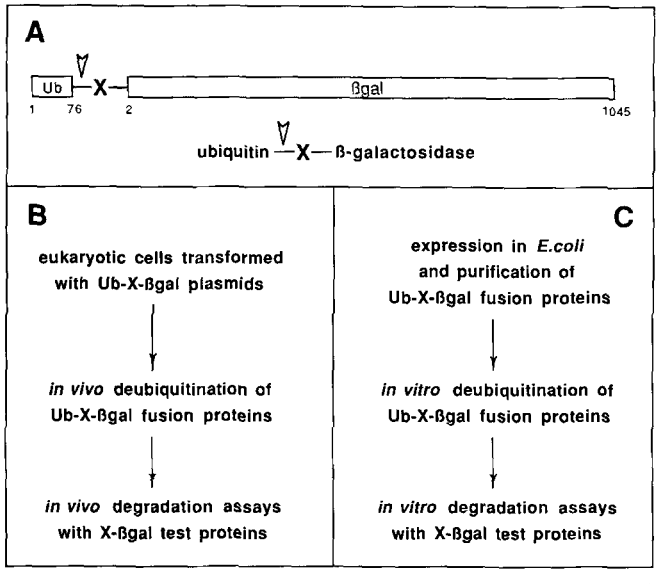

FIG. 1. **The use of ubiquitin-βgal fusions to generate βgal test proteins bearing different amino-terminal residues.** *A*, the residue *X* at the Ub-βgal junction was manipulated (by site-directed mutagenesis of the gene encoding Ub-Met-βgal) to generate 20 Ub-*X*-βgal proteins that differ exclusively at residue *X* (Bachmair *et al.*, 1986). An *arrowhead* indicates the site of the deubiquitinating cleavage that occurs immediately after the last (Gly-76) residue of ubiquitin and yields βgal bearing a new amino-terminal residue, *X*. *B*, the use of Ub-*X*-βgal proteins as *in vivo* proteolytic substrates. *C*, the use of Ub-*X*-βgal proteins as *in vitro* proteolytic substrates.

Amino acid sequencing (by Edman degradation) of deubiquitinated βgal proteins reisolated from either reticulocyte extract or yeast cells showed that, in every case, the proteolytic cleavage occurred precisely at the Ub-βgal junction (Table I), as previously inferred by Bachmair *et al.* (1986) from less extensive evidence. Although sequencing revealed that the amino termini of some *X*-βgal proteins underwent specific modifications (see Table I and below), in no case did these modifications involve proteolytic cleavages beyond the amino-terminal residue *X* (Table I).

All of the deubiquitinated *X*-βgal proteins were metabolically stable in the ATP-depleted reticulocyte extract as judged by SDS-PAGE analysis (Fig. 2, *lanes d–s*, Fig. 3*A*, *lanes b–f*, and data not shown) and from the negligible production of acid-soluble radioactivity in the extract (<0.2% in 2 h at 37 °C; Fig. 4*B*). Thus, preincubation of the Ub-*X*-βgal fusion proteins in the ATP-depleted reticulocyte extract made it possible to generate 20 *X*-βgal test proteins which differed exclusively at the amino-terminal residue *X*.

Half-life of an X-βgal Protein in ATP-supplemented Reticulocyte Extract Is a Function of the X-βgal's Amino-terminal Residue—While all of the 20 *X*-βgal proteins were metabolically stable in the ATP-depleted reticulocyte extract (see above), most of them became short-lived upon addition of ATP to the extract (Table I and Fig. 4, *B–F*). To simplify the discussion, we refer to an amino-terminal residue as stabilizing if the corresponding *X*-βgal is relatively long-lived in the ATP-supplemented extract (<10% degradation in 2 h at 37 °C), and as destabilizing if the degradation of the corresponding *X*-βgal in the extract exceeds 15% under the same conditions (Table I and Fig. 4). While heuristically useful, this classification is oversimplifying in that it ignores relatively small but reproducible and potentially significant differences among the metabolic stabilities of the relatively long-lived *X*-βgal proteins. For example, in this classification, Ile, a destabilizing residue in yeast, is a stabilizing residue in reticulocytes (Table I), although Ile-βgal is degraded significantly (and reproducibly) faster than the other long-lived *X*-βgal proteins in the ATP-supplemented reticulocyte extract (Table I).

The time courses of degradation for several *X*-βgal proteins contained reproducible initial lags (see, for example, the time course for Arg-βgal in Fig. 4*B*). However, semilogarithmic plots of the time courses in Fig. 4 showed that, after the initial lags, the degradation of *X*-βgal in the ATP-supplemented reticulocyte extract obeyed first-order kinetics for at least the first 2 h, making it possible to compare the degradation of different *X*-βgal proteins by comparing their half-lives in the extract (Table I). The range of βgal half-lives in the reticulocyte extract encompasses more than two orders of magnitude (Table I). Half-lives of the metabolically unstable *X*-βgal proteins in reticulocyte extract (Table I) were comparable to the half-lives of other proteolytic substrates (iodinated serum albumin, lysozyme, and cytochrome *c*) in the same

TABLE I

The N-end rule in yeast and in mammalian reticulocytes

The *in vivo* half-lives of *X*-βgal test proteins in the yeast *S. cerevisiae* were determined as previously described (Bachmair *et al.*, 1986). The yeast N-end rule as reported by Bachmair *et al.* (1986) is updated here by inclusion of the four remaining amino acids, Cys, His, Trp, and Asn (see also Bachmair and Varshavsky, 1989). The half-lives of purified, ^{35}S-labeled *X*-βgal test proteins in the ATP-supplemented reticulocyte extract were estimated from semilogarithmic plots of the degradation time courses in Fig. 4 (see main text). The half-lives thus determined were reproducible among different preparations of the extract and of *X*-βgal test proteins (data not shown). Amino acid sequencing of reisolated *X*-βgal proteins is described under "Experimental Procedures." When a mixture of two sequences was obtained, both of the deduced sequences (separated by +) were included into the table.

Residue *X* in Ub-*X*-βgal	Half-life of *X*-βgal		Amino terminus of reisolated *X*-βgal as determined by protein sequencing	
	Yeast (*S. cerevisiae*) *in vivo*	Mammalian reticulocytes *in vitro*	Yeast *in vivo*	Reticulocytes *in vitro*
Val	>20 h	100 h		Val-βgal[b,c]
Met	>20 h	30 h	Met-βgal[a]	Met-βgal[b,c]
Gly	>20 h	30 h		Gly-βgal[b,c]
Pro	>20 h[d]	>20 h[d]		Pro-βgal[d]
Ala	>20 h	4.4 h	Ala-βgal[e]	Ala-βgal[b,f]
Ser	>20 h	1.9 h	—[g]	Ser-βgal[b,f]
Thr	>20 h	7.2 h	Thr-βgal[e]	Thr-βgal[b,f]
Cys	>20 h	1.2 h		[?]-βgal[g,h]
Ile	30 min	20 h	Ile-βgal[e,i]	Ile-βgal[b,c]
Glu	30 min	1.0 h	Arg-Glu-βgal[i]	Glu-βgal + Arg-Glu-βgal[b] Arg-Glu-βgal[f]
His	10 min	3.5 h		His-βgal[b]
Tyr	10 min	2.8 h	Tyr-βgal[e,i]	Tyr-βgal[b]
Gln	10 min	0.8 h	[?]-Glu-βgal[j]	[?]-Glu-β-gal + Glu-βgal[b,k] Arg-Glu-βgal[f]
Asp	3 min	1.1 h	Arg-Asp-βgal[i]	Asp-βgal + Arg-Asp-βgal[b] Arg-Asp-βgal[f]
Asn	3 min	1.4 h	Arg-Asp-βgal[i]	Asn-βgal + Asp-βgal[b] Asn-βgal + Arg-Asp-βgal[f]
Phe	3 min	1.1 h		Phe-βgal[b]
Leu	3 min	5.5 h		Leu-βgal[b]
Trp	3 min	2.8 h		Trp-βgal[b]
Lys	3 min	1.3 h		Lys-βgal[b]
Arg	2 min	1.0 h		Arg-βgal[b]

[a] Determined by radiochemical sequencing (Bachmair *et al.*, 1986).

[b] This *X*-βgal protein was incubated in ATP-depleted reticulocyte extract for 20 min at 37°C before reisolation and sequencing.

[c] This *X*-βgal test protein was incubated in ATP-supplemented reticulocyte extract for 1 h at 37°C before reisolation and sequencing.

[d] In both yeast cells and reticulocyte extract, Ub-Pro-βgal is deubiquitinated approximately 20 times more slowly than are the rest of the Ub-*X*-βgal fusion proteins (see main text). Pro-βgal, the product of slow deuibiquitination of Ub-Pro-βgal, is a long-lived protein in both yeast cells and reticulocyte extract.

[e] The *S. cerevisiae* strain used for expression of this *X*-βgal protein was BWG-9a-1 (*MATα*, *his4*, *ade6*, *ura3*).

[f] This βgal protein was incubated for 2 h at 37°C in ATP-supplemented reticulocyte Fraction II before reisolation and sequencing.

[g] No signal was seen upon sequencing of Ser-βgal reisolated from yeast, strongly suggesting that the protein's amino terminus was blocked. Note that Ser-βgal was not blocked when reisolated from ATP-supplemented reticulocyte extract.

[h] Cys-βgal was incubated in ATP-depleted reticulocyte extract for 30 min at 37°C before reisolation and sequencing. The amino-terminal Cys, unmodified by alkylation before sequencing, could not be identified by the chromatographic procedures used; however, the second and subsequent sequencing steps unambiguously identified the protein as βgal.

[i] The *S. cerevisiae* strain used for expression of this *X*-βgal protein was a mutant (obtained in the background of the BWG-9a-1 strain) in which all of the otherwise short-lived (deubiquitinated) *X*-βgal test proteins are metabolically stable, whereas Ub-Pro-βgal is still short-lived (I. Wünning, A. Bachmair, and A. Varshavsky, unpublished data). This mutant (whose use allowed the isolation of the otherwise short-lived *X*-βgal proteins in quantities sufficient for sequencing) retains both the intact "downstream" degradation pathway and the Ub-*X*-βgal deubiquitinating activity but is impaired in the amino-terminal recognition of at least the *X*-βgal proteins.

[j] The amino-terminal residue of this sequence could not be identified unambiguously with the amount of βgal used (~15 pmol), but, from the data obtained, was most likely Arg. The data clearly identified Glu as the second residue.

[k] The frame-shifted sequence (?)-Glu-βgal was the more abundant (~90%) of the two sequences present. With the amount of βgal used (~15 pmol), the amino-terminal residue of this sequence could not be identified unambiguously but, from the data obtained, was most likely Arg.

extract (Fig. 4, *A* and *B*, and data not shown). These latter test proteins have been used in earlier studies of ubiquitin-dependent protein degradation in reticulocyte extract (reviewed by Hershko, 1988). Recently, at least some of these proteins have been shown to be targeted for degradation via their destabilizing amino-terminal residues (Reiss *et al.*, 1988).

As described above, Ub-Pro-βgal is the only Ub-*X*-βgal fusion protein whose deubiquitination is slow both in yeast and in reticulocyte extract. The product of deubiquitination, Pro-βgal (whose expected amino-terminal sequence has been directly confirmed; Table I), is long-lived in both yeast cells and reticulocyte extract (Table I). The metabolic stability of

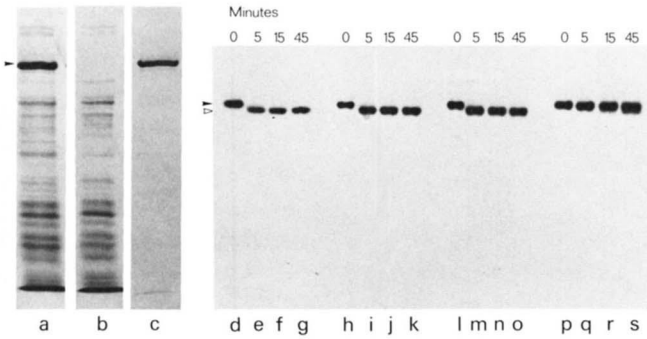

FIG. 2. **Purification of Ub-*X*-βgal fusion proteins and their deubiquitination in reticulocyte extract.** ^{35}S-Labeled proteins in *lanes a–s* were fractionated by SDS-PAGE and detected by fluorography. *Lanes a–c, E. coli* carrying an expression vector which encodes Ub-Gln-βgal were incubated with [^{35}S]methionine, lysed, and the ^{35}S-labeled Ub-Gln-βgal was purified from the extract in one step by affinity chromatography on APTG-Sepharose (see "Experimental Procedures"). *Lane a,* total *E. coli* extract. *Lane b,* flow-through fraction from the APTG-Sepharose column. *Lane c,* same as *lane b* but retained fraction. The same purification procedure was used with the other 19 Ub-*X*-βgal fusion proteins. *Lanes d–s,* equal amounts (1 μg) of the purified, ^{35}S-labeled Ub-*X*-βgal proteins were added separately to 50-μl samples containing 5% (v/v) glycerol, 5 mM $MgCl_2$, 1 mM DTT, 50 mM Tris-HCl, pH 7.5, and 70% (v/v) ATP-depleted reticulocyte extract. Aliquots were taken for analysis by SDS-PAGE after 0, 5, 15, and 45 min of incubation at 37 °C. All 20 of the Ub-*X*-βgal proteins were analyzed this way. The fusion proteins in the four examples shown here are Ub-Met-βgal (*lanes d–g*), Ub-Gln-βgal (*lanes h–k*), Ub-Arg-βgal (*lanes l–o*), and Ub-Pro-βgal (*lanes p–s*). *Filled* and *open arrowheads* indicate the bands of a ~120-kDa Ub-*X*-βgal and a ~112-kDa *X*-βgal, respectively. See "Experimental Procedures" for details.

Pro-βgal is unrelated to the slow deubiquitination of its precursor, Ub-Pro-βgal: when Pro-βgal was purified from ATP-depleted reticulocyte extract by affinity chromatography on APTG-agarose (see "Experimental Procedures"), and then added back to ATP-supplemented reticulocyte extract, it remained as long-lived as the Pro-βgal derived from Ub-Pro-βgal directly in the extract (data not shown).

While in yeast the unprocessed (non-deubiquitinated) Ub-Pro-βgal is a short-lived protein, with a half-life of ~7 min (Table I), it is, aside from its slow deubiquitination, a metabolically stable protein in the ATP-supplemented reticulocyte extract (data not shown). We do not understand the reason for this difference between the fates of Ub-Pro-βgal in yeast cells and in reticulocyte extract.

Degradation of X-βgal Proteins in Reticulocyte Fraction II Is Ubiquitin-dependent—To remove free ubiquitin from the reticulocyte extract, it was fractionated by DEAE-chromatography to yield a fraction, called Fraction II (Hershko, 1988), which lacks most of the extract's free ubiquitin but retains the deubiquitinating activity and the ability to carry out ubiquitin-dependent protein degradation if supplemented with purified ubiquitin and ATP (see "Experimental Procedures"). The degradation of *X*-βgal proteins (where *X* is Met, Val, Ser, Gln, Glu, Asp, Asn, Cys, Ile, Phe, or Arg), and also of *in vitro* iodinated serum albumin and lysozyme, was examined in Fraction II with and without added ATP and free ubiquitin. In agreement with earlier evidence (reviewed by Hershko and Ciechanover, 1986), the absolute rates of protein degradation in the ATP- and ubiquitin-supplemented Fraction II were significantly lower than those in the unfractionated, ATP-supplemented reticulocyte extract. Nevertheless, the degradation of *X*-βgal in Fraction II was still a function of the protein's amino-terminal residue and was completely

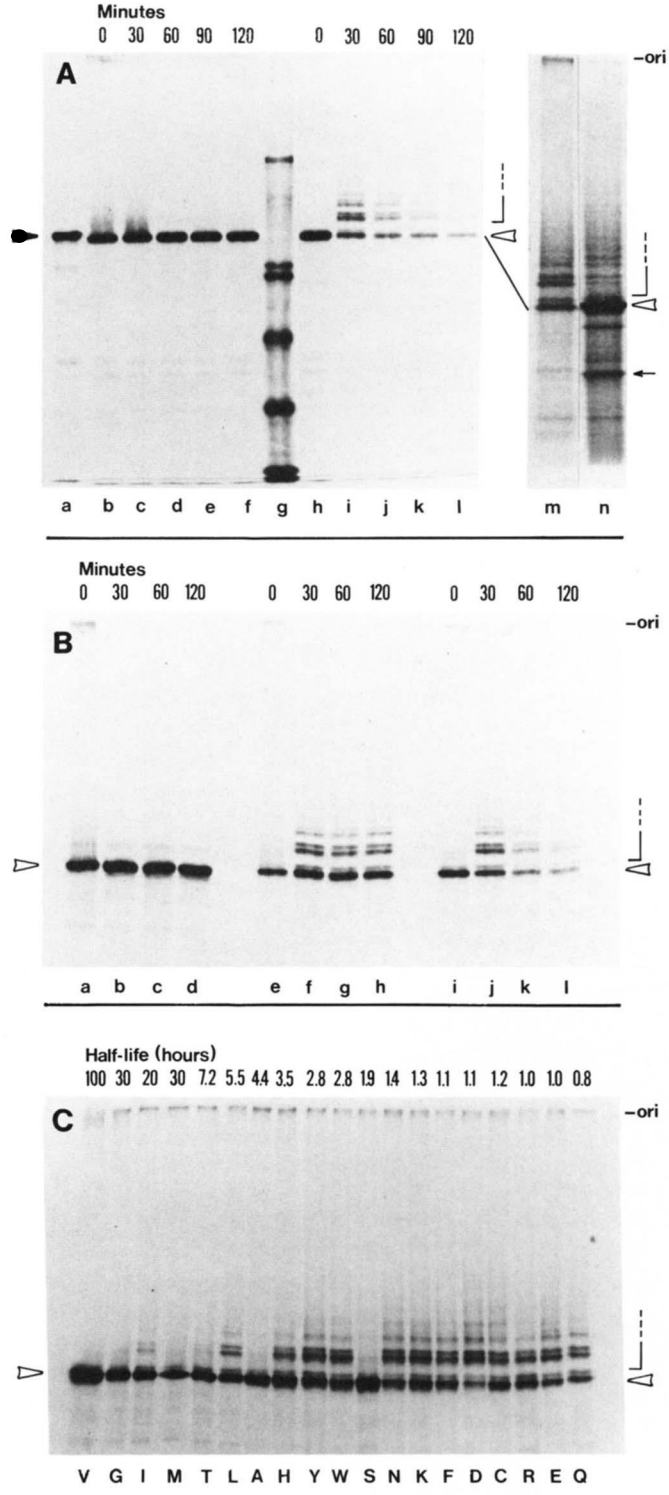

FIG. 3. **Electrophoretic analysis of *X*-βgal degradation in reticulocyte extract.** The ^{35}S-labeled βgal test proteins in *panels A–C* were fractionated by SDS-PAGE and detected by fluorography. The *filled arrowhead* in *A* indicates the band of the initial, ~120-kDa Ub-Glu-βgal fusion protein in *lane a*. The *open arrowheads* in *panels A–C* indicate the bands of (deubiquitinated) ~112-kDa *X*-βgal test proteins. The *half-open square brackets* in *panels A–C* indicate the bands of multiply ubiquitinated βgal. The *arrow by lane n* indicates the ~90-kDa cleavage product of *X*-βgal that accumulates during *X*-βgal degradation in yeast cells (Bachmair *et al.*, 1986) but not in reticulocyte extract (see main text). *Panel A,* the Glu-βgal test protein (produced via deubiquitination of Ub-Glu-βgal) was incubated in the reticulocyte extract in the absence (*lanes b–f*) or in the presence (*lanes h–l*) of ATP, and aliquots were taken for SDS-PAGE analysis after 0, 30, 60, 90, and 120 min at 37 °C. *Lane a* contains the initial Ub-Glu-βgal fusion protein. *Lane g* contains ^{14}C-labeled molecular

dependent on the addition of ATP and ubiquitin (data not shown). Except where noted, the *in vitro* system used in our further experiments was the unfractionated reticulocyte extract because it is both more active in selective protein degradation and less perturbed (processed) than Fraction II.

Short-lived X-βgal Proteins Are Multiply Ubiquitinated—Metabolically stable *X*-βgal proteins did not undergo either limited proteolysis or extensive ubiquitination during their incubation in the ATP-supplemented reticulocyte extract, as indicated by SDS-PAGE analysis, measurements of acid-soluble radioactivity, and direct sequencing of *X*-βgal proteins reisolated from the extract (Figs. 3, *B* and *C*, and 4, and Table I). In contrast, the degradation of short-lived *X*-βgal proteins in either the ATP-supplemented reticulocyte extract or the ATP- and ubiquitin-supplemented Fraction II was accompanied by the formation of a set of higher molecular weight, *X*-βgal-containing species which was electrophoretically indistinguishable from the previously observed set of ubiquitin-βgal conjugates formed during the *in vivo* degradation of short-lived *X*-βgal proteins in yeast (Fig. 3*A*, *lanes m* and *n*; and *B* and *C*; see also Bachmair *et al.*, 1986). Direct mapping of ubiquitin moieties within multiply ubiquitinated *X*-βgal proteins has confirmed a close similarity, if not the identity of multiubiquitin arrangements in *X*-βgal isolated from yeast cells and from reticulocyte extract (Chau *et al.*, 1989; see also "Discussion").

No discrete βgal cleavage products were detectable by SDS-PAGE during the degradation of *X*-βgal proteins in reticulocyte extract (Fig. 3 and data not shown). In particular, no ~90-kDa, metabolically stable βgal cleavage product that accumulates during the degradation of short-lived *X*-βgal proteins in yeast (Bachmair *et al.*, 1986) was observed during *X*-βgal degradation in reticulocyte extract (Fig. 3*A*, *lanes h–n*, and *B*). The degradation of an *X*-βgal is apparently highly processive, with intermediates (other than multiply ubiquitinated βgal) that are not detectable by SDS-PAGE analysis.

With three exceptions, the steady-state levels of ubiquitin-βgal conjugates in reticulocyte extract closely correlated with the half-lives of corresponding *X*-βgal proteins (Fig. 3*C*). The exceptions, Ala-, Ser-, and Thr-βgal, attained significantly lower steady-state levels of ubiquitin-βgal conjugates than could be expected from comparisons with other *X*-βgal proteins of approximately equal metabolic stability (Fig. 3*C*).

The three relatively long-lived *X*-βgal proteins (Met-, Gly-, and Ile-βgal), but not the longest-lived Val-βgal, gave rise to low levels of steady-state ubiquitination (Fig. 3, *B* and *C*),

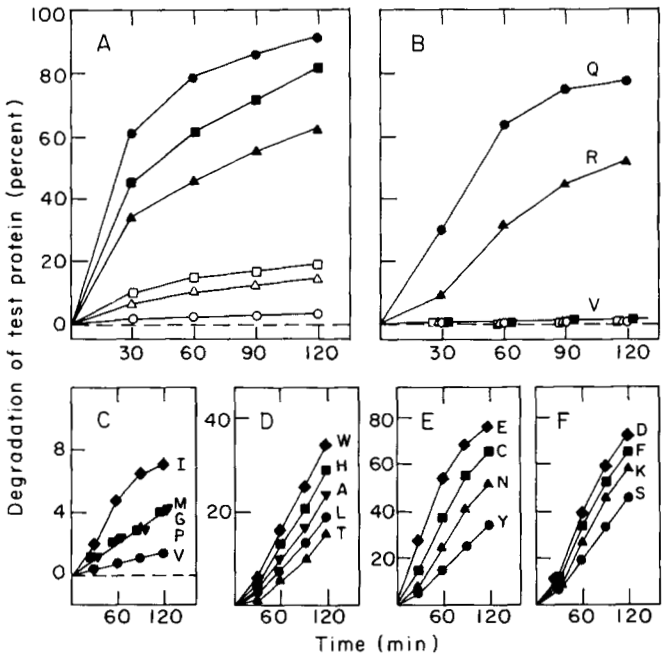

FIG. 4. **ATP-dependent degradation of test proteins in reticulocyte extract.** Degradation of radioactively labeled test proteins was assayed by measuring acid-soluble radioactivity in either ATP-supplemented (*closed symbols*) or ATP-depleted (*open symbols*) reticulocyte extract. *Panel A*, test proteins were either bovine serum albumin (*circles*), cytochrome *c* from *S. cerevisiae* (*squares*), or hen lysozyme (*triangles*), all of which were labeled with ^{125}I *in vitro* using the chloramine-T method (see "Experimental Procedures"). *Panels B–F*, test proteins were the 20 *X*-βgal proteins produced by deubiquitination of the corresponding Ub-*X*-βgal fusion proteins that had been metabolically labeled with ^{35}S. The *letters* marking each of the time courses refer, in single-letter abbreviations (see the legend to Fig. 3), to the amino-terminal residue *X* in *X*-βgal. For all of the [^{35}S] methionine-labeled *X*-βgal proteins, the amounts of acid-soluble radioactivity released during a 2-h incubation at 37 °C in the ATP-depleted reticulocyte extract were below 0.2% of the input radioactivity (*panel B* and data not shown). See "Experimental Procedures" for details.

consistent with the fact that the half-lives of these proteins in reticulocyte extract were significantly shorter than that of Val-βgal (Table I).

Primary and Secondary Destabilizing Residues—Earlier studies of ubiquitin-dependent protein degradation in reticulocyte extract have shown that the degradation of some proteolytic substrates in this system requires the presence of tRNA (Ferber and Ciechanover, 1986). The N-end rule (Bachmair *et al.*, 1986) provides a mechanistically straightforward explanation for the above observations. In both bacteria and eukaryotes there exist aminoacyl-tRNA-protein transferases, which catalyze posttranslational conjugation of specific amino acids to the mature amino termini of acceptor proteins (Kaji *et al.*, 1965; Soffer, 1980; Deutch, 1984). This posttranslational conjugation of amino acids to proteins is greatly enhanced in a stressed or regenerating tissue, for instance, in physically injured axons of neurons (Ingoglia *et al.*, 1983; Shyne-Athwal *et al.*, 1986). We have previously suggested that certain amino-terminal residues in proteins may not be directly destabilizing as such but become so only through their ability to be conjugated to other, *primary* destabilizing residues (Bachmair *et al.*, 1986). Later work (Ferber and Ciechanover, 1987) supported this model by showing that several proteolytic substrates, whose only obvious common feature is the presence of an acidic amino-terminal residue (Glu or Asp), apparently required amino-terminal conjugation of Arg for their degra-

mass markers (Amersham Corp.; 200, 100, 92.5, 69 and 46 kDa, respectively). *Lanes m* and *n*, comparison of the multiply ubiquitinated βgal species formed during *in vitro* degradation of Gln-βgal in the reticulocyte extract (*lane m*) with the analogous ubiquitin-βgal conjugates formed during *in vivo* degradation of Gln-βgal in the yeast *S. cerevisiae* (*lane n*). In the latter case, the [^{35}S]methionine-labeled βgal and its ubiquitinated derivatives were isolated from yeast extract by immunoprecipitation with a monoclonal antibody to βgal (Bachmair *et al.*, 1986). *B*, Val-βgal (*lanes a–d*), Lys-βgal (*lanes e–h*), and Phe-βgal (*lanes i–l*) were incubated in ATP-supplemented reticulocyte extract, and aliquots were taken for SDS-PAGE analysis after 0, 30, 60, and 120 min at 37 °C. *C*, equal amounts (1 μg) of 19 *X*-βgal proteins were incubated separately in ATP-supplemented reticulocyte extract for 30 min at 37 °C, followed by SDS-PAGE analysis. See "Experimental Procedures" for details. A letter below each lane refers, in a single-letter abbreviation, to the amino-terminal residue *X* in the corresponding *X*-βgal. A number *above* each lane refers, in hours, to the approximate half-life of the corresponding *X*-βgal in the reticulocyte extract (see Table I). The order of lanes was chosen to approximate the order of metabolic stabilities of *X*-βgal test proteins in the reticulocyte extract (Fig. 4 and Table I). Single-letter amino acid abbreviations: *A*, Ala; *C*, Cys; *D*, Asp; *E*, Glu; *F*, Phe; *G*, Gly; *H*, His; *I*, Ile; *K*, Lys; *L*, Leu; *M*, Met; *N*, Asn; *P*, Pro; *Q*, Gln; *R*, Arg; *S*, Ser; *T*, Thr; *V*, Val; *W*, Trp; *Y*, Tyr.

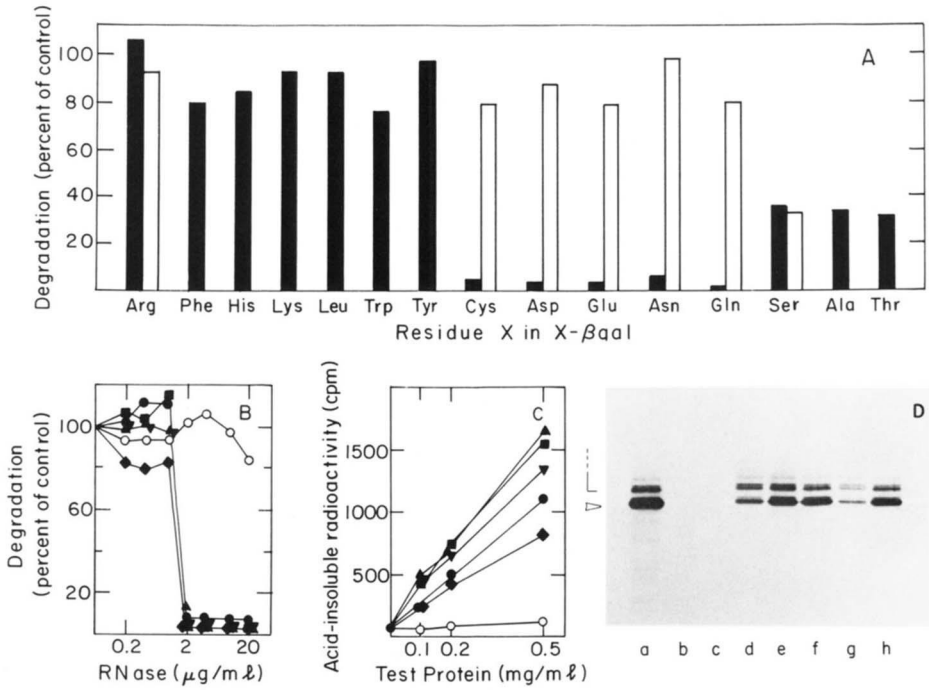

FIG. 5. **Primary and secondary destabilizing amino acids.** *Panel A,* metabolically unstable *X*-βgal proteins whose initial amino-terminal residues are indicated *below the bars,* were incubated in ATP-supplemented reticulocyte extract which had been pretreated for 45 min at 37 °C with 3 units/ml of RNase A-agarose (Sigma) and centrifuged to remove the immobilized RNase A before the addition of Ub-*X*-βgal. *Closed bars* indicate the extent of *X*-βgal degradation produced by the RNase A-pretreated extract. Identical results were obtained by preincubating the extract with 20 μg/ml of soluble RNase A (*panel A* and data not shown). *Open bars* correspond to otherwise identical reactions in which the extract that had been pretreated with RNase A-agarose was supplemented with 50 μg/ml of the purified total tRNA from bovine liver and with 1500 units/ml of a RNase inhibitor RNasin (Promega Biotech; control experiments showed that RNasin alone had no effect on the same reactions). Degradation of these [^{35}S]methionine-labeled *X*-βgal proteins was assayed by measuring the amount of acid-soluble ^{35}S radioactivity after 2 h at 37 °C and is presented relative to the degradation of *X*-βgal in corresponding control reactions, carried out in parallel and otherwise identically but without RNase A pretreatment or addition of tRNA. Pretreatment of reticulocyte extract with RNase A did not inhibit deubiquitination of any of the Ub-*X*-βgal test proteins (data not shown). *Panel B,* inhibition of *X*-βgal degradation in reticulocyte extract as a function of the extent of RNase A treatment. Reactions were carried out with ^{35}S-labeled *X*-βgal proteins as described in *panel A* except that soluble (non-immobilized) RNase A (50 units/mg; Sigma) was used, and its concentration in the extract was varied from 0 to 20 μg/ml. Arg-βgal (○——○); Cys-βgal (●——●); Asp-βgal (■——■); Glu-βgal (▲——▲); Asn-βgal (◆——◆); Gln-βgal (▼——▼). *Panel C,* arginylation of *X*-βgal in reticulocyte extract. Unlabeled *X*-βgal proteins were incubated at the indicated concentrations in dialyzed, ATP-supplemented reticulocyte extract at 37 °C in the presence of 6.6 μM [^{3}H]arginine, and the incorporation of [^{3}H]arginine into hot trichloroacetic acid-insoluble material was assayed as a function of time. The same (but unlabeled) *X*-βgal proteins and the same designations were used in *panel B* above. *Panel D,* in *lane a,* dialyzed, ATP-supplemented, reticulocyte extract containing 6.6 μM unlabeled arginine and 0.25 mg/ml of [^{35}S]Arg-βgal was incubated for 30 min at 37 °C prior to SDS-PAGE analysis. In the other lanes (*b–h*), dialyzed, ATP-supplemented reticulocyte extract containing 6.6 μM [^{3}H]arginine, and either no *X*-βgal (*lane b*) or 0.25 mg/ml of unlabeled Arg-βgal (*lane c*), Cys-βgal (*lane d*), Asp-βgal (*lane e*), Glu-βgal (*lane f*), Asn-βgal (*lane g*), and Gln-βgal (*lane h*) were incubated as in *lane a* prior to SDS-PAGE analysis. The *arrowhead* indicates the band of (deubiquitinated) ~112-kDa *X*-βgal. The *half-open square brackets* indicate the bands of multiply ubiquitinated βgal. See "Experimental Procedures" for details.

dation. In the experiments described below, we used the complete set of metabolically unstable *X*-βgal proteins in the reticulocyte extract and in yeast cells to examine rigorously and systematically the role of amino acid conjugation in protein degradation. In doing so, we identified Cys as a previously unknown *secondary* destabilizing residue in reticulocytes (in addition to Asp and Glu) and discovered a new class of *tertiary* destabilizing residues.

Preincubation of the reticulocyte extract with RNase A completely prevented the degradation of five *X*-βgal proteins (Cys-, Asp-, Glu-, Asn-, and Gln-βgal), partially inhibited the degradation of Ala-, Ser-, and Thr-βgal, but did not significantly affect the degradation of the remaining seven short-lived *X*-βgal proteins (His-, Lys-, Arg-, Phe-, Leu-, Trp-, and Tyr-βgal) (Fig. 5, *A* and *B*). Preincubation of the extract with 2 μg/ml of RNase A under the above conditions was sufficient to prevent the degradation of Cys-, Asp-, Glu-, Gln-, and Asn-βgal (Fig. 5*B*), whereas much greater amounts of RNase A (>10 μg/ml) were needed for an even partial inhibition of degradation of Ala-, Ser-, and Thr-βgal (data not shown). Supplementing RNase-treated extract with either total bovine tRNA or total RNA isolated from reticulocyte extract almost completely restored the degradation of Cys-, Asp-, Glu-, Gln-, and Asn-βgal but did not relieve the partial inhibition of the degradation of Ala-, Ser-, and Thr-βgal (Fig. 5*A* and data not shown). Thus, the degradation of Ala-, Ser-, and Thr-βgal apparently does not require tRNA and hence does not require amino acid conjugation; this conclusion is supported also by the results of amino acid sequencing of these test proteins (see Table I) and by the results of dipeptide inhibition experiments (see below). Amounts of RNase A that inhibited the tRNA-dependent degradation of a protein such

as Asp-βgal also inhibited its ubiquitination (data not shown).

To detect amino acid conjugation to X-βgal, we incubated unlabeled X-βgal proteins in the reticulocyte extract supplemented with ATP, an ATP-regenerating system, and various ^{3}H-amino acids. Efficient incorporation of added [^{3}H]arginine into hot trichloroacetic acid-insoluble material was observed with samples containing Cys-, Asp-, Glu-, Gln-, and Asn-βgal (Fig. 5C). Analysis of the same samples by SDS-PAGE indicated that the [^{3}H]arginine-derived radioactivity comigrated exclusively with the X-βgal proteins and their ubiquitin conjugates in the sample (Fig. 5D). At the same time, virtually no incorporation of [^{3}H]arginine into hot trichloroacetic acid-insoluble material occurred in the otherwise identical samples that either lacked X-βgal proteins or contained Ala-, Ser-, Thr-, Arg-, or Lys-βgal (Fig. 5, C and D and data not shown). At least the bulk of [^{3}H]arginine has been shown to be conjugated specifically to the amino termini of X-βgal proteins (Chau *et al.*, 1989). Although histidinylation has been suggested to be involved in the degradation of some proteolytic substrates (Ferber and Ciechanover, 1987), we detected no incorporation of added [^{3}H]histidine into any of the five X-βgal proteins whose degradation was tRNA-dependent in the reticulocyte extract, nor into any of several other X-βgal proteins, including Ala-, Ser-, and Thr-βgal. We also detected no incorporation of [^{3}H]Lys, Phe, Leu, Tyr, Trp and Ser into hot trichloroacetic acid-insoluble material in analogous experiments with X-βgal proteins in reticulocyte extract (data not shown).

Amino acid sequencing of Asp-βgal and Glu-βgal reisolated from either the ATP-depleted reticulocyte extract, the ATP-supplemented Fraction II or *S. cerevisiae* cells directly confirmed, in both yeast and reticulocytes, that the amino termini of Asp-βgal and Glu-βgal were conjugated to Arg (Table I). The observed conjugation of Arg to Cys-βgal, as well as to Asp- and Glu-βgal in reticulocyte extract (Fig. 5) is consistent with the known substrate specificity of the isolated mammalian arginyl-tRNA-protein transferase that catalyzes these reactions (Soffer, 1980). We conclude that Asp and Glu are secondary destabilizing residues in both yeast and mammalian reticulocytes, whereas Cys is a secondary destabilizing residues in reticulocyte extract but not in yeast cells where it is a stabilizing residue. Although the tRNA dependence of the degradation of Asn-βgal and Gln-βgal in reticulocyte extract (Fig. 5, A and B) was consistent with Asn and Gln being secondary destabilizing residues as well, further analysis has shown that these residues belong to a new class.

Tertiary Destabilizing Residues—Amino acid sequencing of Asn-βgal and Gln-βgal reisolated from either the ATP-depleted reticulocyte extract, the ATP-supplemented reticulocyte Fraction II, or yeast (*S. cerevisiae*) cells indicated that Asn and Gln at the amino termini of these proteins were deamidated to Asp and Glu, respectively, apparently before their conjugation to Arg (Table I). Consistent with this result, the isolated mammalian arginyl-tRNA-protein transferase efficiently arginylates amino-terminal Asp and Glu but does not arginylate amino-terminal Gln (Soffer, 1980).

In control experiments, we ruled out the possibility that the amino-terminal Gln or Asn could be undergoing nonenzymatic deamidation during the isolation of X-βgal from either *E. coli*, yeast cells, or reticulocyte extract. Site-directed mutagenesis was used to modify the *E. coli* expression vector encoding Ub-Gln-βgal such that the last four codons of the ubiquitin reading frame specified Ile-Glu-Gly-Arg, the sequence recognized by Factor Xa, one of the proteases in the blood clotting cascade (see "Experimental Procedures"). Digestion of the purified Ub-(Xa)-Gln-βgal protein with purified Factor Xa resulted in an X-βgal-sized product which, after a mock incubation in the absence of reticulocyte extract, followed by amino acid sequencing, was identified as Gln-βgal (see "Experimental Procedures"). On the other hand, when the same incubation was carried out in reticulocyte extract, the amino-terminal Gln of Gln-βgal was found to be converted to Glu. This conversion was prevented by treating reticulocyte extract with the thiol blocking reagent *N*-ethylmaleimide prior to the addition of Gln-βgal (data not shown).

We conclude that the deamidation of Gln-βgal and Asn-βgal after incubation in reticulocyte extract is due to enzymatic deamidation and not to the later steps of reisolation and handling of the test protein prior to amino acid sequencing. We therefore suggest that Asn and Gln be designated tertiary destabilizing residues, *i.e.* amino-terminal residues that are not destabilizing as such but become so only through their ability to be converted, via enzymatic deamidation, into the secondary destabilizing residues Asp and Glu.

The existence of tertiary destabilizing residues in both yeast and mammalian cells implies the existence of a deamidase(s) specific for the amino-terminal Asn and Gln residues. An enzyme with this specificity remains to be directly identified. We have recently isolated a *S. cerevisiae* mutant in which Asn-βgal (but not the other short-lived X-βgal proteins) is metabolically stable, suggesting a mutation in a gene for the deamidase.[2] To our knowledge, the only previously characterized example of enzymatic deamidation in proteins is the deamidation of specific Gln residues in bacterial chemotaxis receptors (Kehry *et al.*, 1983; Terwilliger and Koshland, 1984).

Metabolic Instability of X-βgal Bearing a Destabilizing Amino-terminal Residue Is Independent of the Ubiquitin Fusion Technique Used to Generate X-βgal—Purified Gln-βgal produced *in vitro* using the Factor Xa technique (see above and "Experimental Procedures") was as short-lived upon its addition to the ATP-supplemented reticulocyte extract as was Gln-βgal produced via deubiquitination of the Ub-Gln-βgal protein in the same extract (Table I and data not shown). Furthermore, the degradation of the Factor Xa-produced Gln-βgal was accompanied by the formation of multiply ubiquitinated βgal species indistinguishable from those observed with Gln-βgal that had been produced from Ub-Gln-βgal (Fig. 3, B and C and data not shown). These results ruled out the possibility that the temporal and spatial proximity between deubiquitination of Ub-X-βgal and degradation of the resulting X-βgal might have masked some additional role of the deubiquitination step in the subsequent degradation of X-βgal.

Three N-end-recognizing Activities in Reticulocytes—In a recent work, Reiss *et al.* (1988) have shown that certain dipeptides (as well as other carboxyl-terminal amino acid derivatives such as amino acid esters) specifically inhibit the degradation of some proteolytic substrates in ATP-supplemented reticulocyte extract. In particular, dipeptides having a basic amino-terminal residue (Arg, Lys, or His) inhibited both the degradation and ubiquitination of proteolytic substrates such as lysozyme and oxidized RNase A that have a basic residue (Lys) at their amino termini (Reiss *et al.*, 1988). On the other hand, dipeptides having a bulky hydrophobic amino-terminal residue (Phe, Leu, Trp, or Tyr) inhibited both the degradation and ubiquitination of proteolytic substrates such as β-lactoglobulin and pepsinogen which have a bulky hydrophobic residue (Leu) at their amino termini.

These and other findings indicated that dipeptides competitively inhibit the binding of a proteolytic substrate's amino-terminal residue (a *primary destabilizing* residue, in the N-

[2] R. Baker and A. Varshavsky, unpublished data.

end rule terminology) to the previously defined E3 component of ubiquitin-protein ligase. Thus, as suggested earlier (Bachmair *et al.*, 1986), E3 is the N-end-recognizing protein or at least a component thereof. We used the approach of Reiss *et al.* (1988) together with the complete set of short-lived *X*-βgal proteins, to determine systematically the number of distinct N-end-recognizing (E3) activities in reticulocyte extract. In doing so, we identified a previously unknown N-end-recognizing activity specific for Ala, Ser, and Thr, which are primary destabilizing residues in reticulocytes but *stabilizing* residues in yeast (Table I).

As shown in Table II and Fig. 6*D*, the dipeptide His-Ala specifically inhibited the degradation of *X*-βgal proteins whose amino-terminal residues were either basic (Arg, Lys, His) or those (Asp, Glu, Asn, Gln, Cys) which acquired a basic amino-terminal residue as the result of enzymatic conjugation of Arg (see Figs. 7 and 8, and Table I). The dipeptide Trp-Ala specifically inhibited the degradation of *X*-βgal proteins bearing bulky hydrophobic residues (Phe, Leu, Trp, or Tyr) at their amino termini (Table II and Fig. 6*C*). Neither His-Ala nor Trp-Ala, nor an equimolar mixture of these dipeptides inhibited the degradation of Ala-, Ser-, and Thr-βgal, the only *X*-βgal proteins (in addition to Cys-βgal) that are short-lived in reticulocytes but long-lived in yeast (Table II, Figs. 6, *C* and *D*, and 7, and data not shown). However, the dipeptides Ser-Ala and Ser-Gly did inhibit the degradation of Ala-, Ser-, and Thr-βgal in reticulocyte extract, although the extent of inhibition was smaller than that observed with His-Ala, Trp-Ala, and their cognate *X*-βgal proteins (Table II and Fig. 6, *E* and *F*). The relatively weak inhibition of the degradation of Ser-βgal by Ser-Ala and Ser-Gly could be augmented by increasing the inhibitor size to a tripeptide such as Ser-Gly-Gly (Fig. 6*F*). None of these peptides inhibited the degradation of Lys-βgal and Phe-βgal which have a basic and a bulky hydrophobic residue, respectively, at their amino termini (Table II, Fig. 6*E*, and data not shown). The relatively weak interaction of Ser-Ala and Ser-Gly with the putative binding site for Ala-, Ser-, and Thr-βgal in a N-end-recognizing (E3) protein may account for our inability to detect specific inhibition of the degradation of test proteins by Ala-Ala, Ala-Ser, and Thr-Ala dipeptides (Fig. 6*F* and data not shown). This interpretation is also consistent with the fact that Ser-βgal is a shorter-lived protein in reticulocyte extract than either Ala-βgal or Thr-βgal (Table I). In agreement with the earlier evidence (Reiss *et al.*, 1988), amounts of a dipeptide that inhibited the degradation of a specific set of *X*-βgal proteins also inhibited their ubiquitination in reticulocyte extract (data not shown).

The dipeptide Ile-Ala did not inhibit the degradation of either Ser-βgal or Lys-βgal but did weakly inhibit the degradation of Phe-βgal whose amino-terminal residue is bulky and hydrophobic (Fig. 6*B*). This result is consistent with the fact

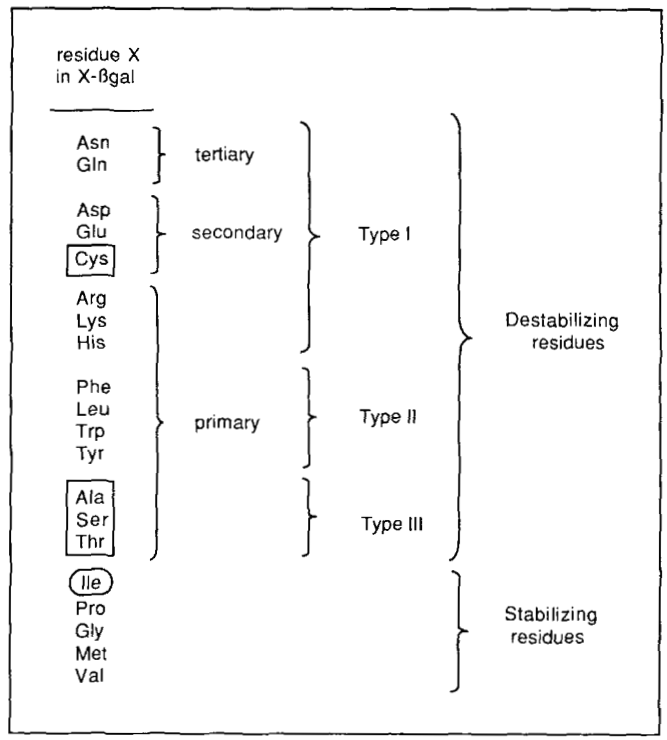

FIG. 7. **Structure of N-end rule.** The terms primary, secondary, and tertiary destabilizing amino-terminal residues have been defined in the main text and in Fig. 8. *Type I, II,* and *III* refer to the specific classes of destabilizing amino-terminal residues that are recognized (either directly or indirectly; see Fig. 8) by distinct types of the N-end-recognizing (E3) activity in reticulocytes, as discussed in the main text. Unlike the above classification categories, the distinction between stabilizing and destabilizing amino-terminal residues is in part heuristic because *X*-βgal proteins with different "stabilizing" amino-terminal residues have detectably different metabolic stabilities (see Table I and "Discussion"). The N-end rule shown is that of mammalian (rabbit) reticulocytes. The yeast (*S. cerevisiae*) N-end rule differs in having Ala, Ser, Thr, and Cys (*boxed*) as stabilizing residues and Ile (*rounded frame*) as a destabilizing residue (see Table I).

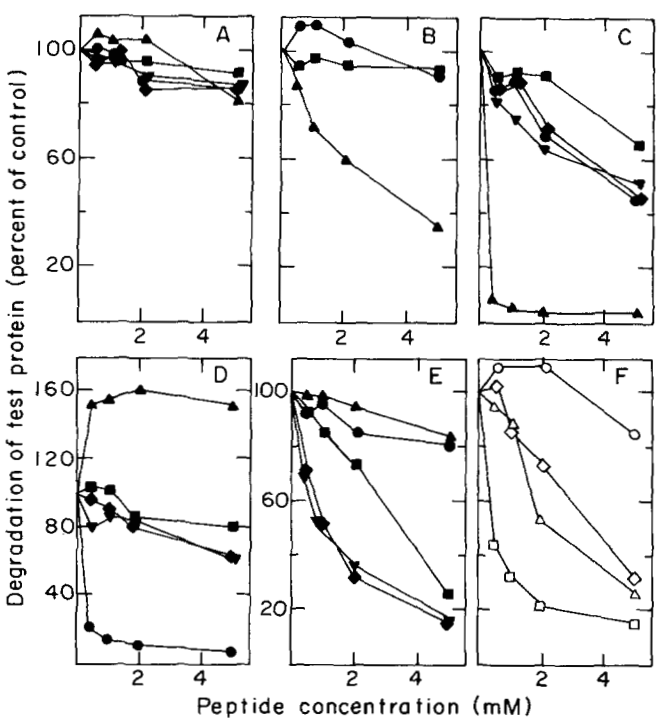

FIG. 6. **Amino terminus-specific inhibition of βgal degradation by dipeptides.** ^{35}S-Labeled Lys-βgal (●——●), Phe-βgal (▲——▲), Ala-βgal (▼——▼), Thr-βgal (♦——♦), and Ser-βgal (■——■) were incubated in ATP-supplemented reticulocyte extract in the presence of bestatin at 40 μg/ml (Reiss *et al.*, 1988) and increasing concentrations of specific peptides (obtained from Bachem Bioscience, Philadelphia, PA). Degradation of *X*-βgal was assayed by measuring the amount of acid-soluble radioactivity after 2 h at 37 °C and is plotted against the degradation of βgal in the otherwise identical control reactions carried out in the absence of added peptides. The peptides used were Val-Ala (*A*), Ile-Ala (*B*), Trp-Ala (*C*), His-Ala (*D*), and Ser-Ala (*E*). In *F*, the degradation of ^{35}S-labeled Ser-βgal was assayed in the presence of increasing amounts of the following peptides: Ser-Ala (△——△), Ala-Ser (○——○), Ser-Gly (◇——◇), and Ser-Gly-Gly (□——□). See "Experimental Procedures" for details.

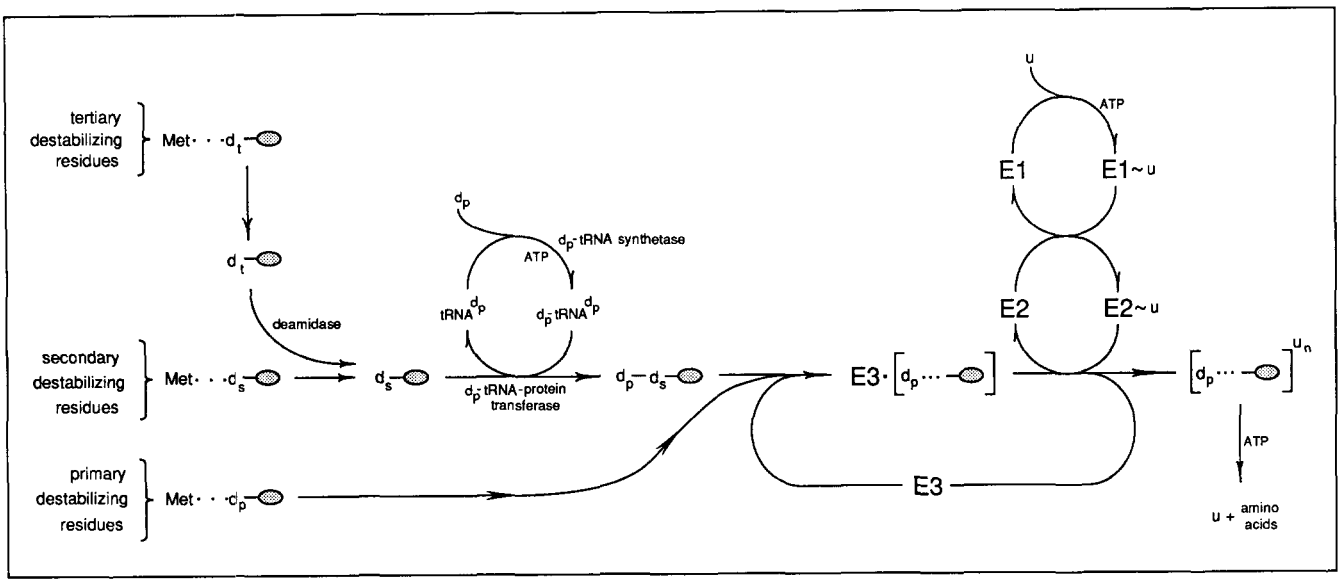

FIG. 8. **The N-end rule pathway.** The designations used are: d_t, *tertiary* destabilizing amino-terminal residues Asn or Gln, which are destabilizing through their ability to be converted, via selective deamidation, into the *secondary* destabilizing residues, Asp or Glu, respectively; d_s, *secondary* destabilizing amino-terminal residues, Asp or Glu (and also Cys in reticulocytes; see Table I and Fig. 7), which are destabilizing through their ability to be conjugated to a *primary* destabilizing residue, d_p (largely, but not necessarily exclusively, Arg). The *dotted oval* denotes the rest of a protein molecule. The N-end-recognizing protein is denoted as E3, since the properties of a mammalian protein E3, whose presence is required for ubiquitination of proteolytic substrates by E2 enzymes *in vitro* (Hershko, 1988) strongly suggest that E3 is the N-end-recognizing protein or at least a component thereof (see main text). E1 and E2, ubiquitin-activating and ubiquitin-conjugating enzymes, respectively. *u*, ubiquitin. The designation $[\cdots]^{u_n}$ denotes the targeted protein which contains a multiubiquitin chain of *n* ubiquitin moieties branch conjugated to each other via a Gly-76/Lys-48 isopeptide bond between adjacent ubiquitins, and joined (via the carboxyl-terminal Gly-76 of the first ubiquitin moiety in the chain) to a specific Lys residue of the target protein (Chau *et al.*, 1989; see also Bachmair and Varshavsky (1989), and the main text). The N-end rule pathway starts when a newly formed protein is proteolytically processed *in vivo* to expose one of the three distinct types of destabilizing amino-terminal residues, d_t, d_s, or d_p. For instance, if an initially processed protein bears one of the two tertiary (d_t) destabilizing amino-terminal residues (Asn or Gln), it is selectively deamidated at the amino terminus, yielding the amino-terminal d_s residue (Asp or Glu, respectively), which is then conjugated to a primary destabilizing residue, d_p (see above). The amino-terminal d_p residue is bound by the N-end-recognizing protein (E3), and, if the second determinant of the amino-terminal degradation signal is present as well (see Bachmair and Varshavsky (1989), and the main text), the protein is targeted for multiple ubiquitination and subsequent selective degradation. The deamidation route is bypassed when the exposed amino-terminal residue in an initially processed protein is a secondary destabilizing residue, d_s (see Fig. 7). If the initial proteolytic processing yields a primary destabilizing residue, d_p, at the protein's amino terminus, the protein is targeted for ubiquitination directly, bypassing the amino acid conjugation step as well. Although E3 is shown to recycle after the ubiquitination step, other possibilities are also not precluded by the presently available evidence (see Bachmair and Varshavsky, 1989). Note that by not specifying the positions of relevant destabilizing residues within a protein, this scheme implicitly incorporates the possibility of proteolytic processing of either newly formed or preexisting proteins that could expose destabilizing residues far away from the protein's initial amino terminus. See main text for additional details and discussion.

that Ile, also a bulky hydrophobic residue, is a "borderline" stabilizing residue in reticulocytes and a destabilizing residue in yeast (see Table I, Fig. 7, and discussion above). The dipeptide Val-Ala did not inhibit the degradation of either Lys-, Phe-, Ser-, Ala-, or Thr-βgal (Fig. 6*A*), consistent with the fact that Val is the strongest stabilizing residue in the reticulocyte N-end rule (Table I).

The dipeptide His-Ala, while inhibiting the degradation of *X*-βgal proteins with basic amino-terminal residues, *stimulated* the degradation of *X*-βgal with bulky hydrophobic amino-terminal residues (Fig. 6*D* and Table II). One explanation of this effect is that dipeptides with basic amino-terminal residues inhibit the degradation of abundant endogenous substrates with basic amino termini in the reticulocyte extract, and thereby reduce competition for some limiting component of the proteolytic pathway. Since His-Ala did not stimulate the degradation of Ala-, Ser-, or Thr-βgal (Fig. 6*D*), the hypothetical limiting component of the proteolytic pathway is apparently not limiting in the degradation of this specific subset of short-lived *X*-βgal proteins.

These results (Table II and Fig. 6) demonstrated the existence of three functional classes of primary destabilizing residues, and implied the presence of three distinct types of N-end-recognizing (E3) activities in mammalian reticulocytes. Two of these activities (those specific for bulky hydrophobic and basic amino-terminal residues) have been inferred previously by Reiss *et al.* (1988). The third activity, specific for *small uncharged* destabilizing amino-terminal residues, Ala, Ser, and Thr, was identified in the present work.

DISCUSSION

Structure of the N-End Rule—The hierarchical organization of the N-end rule that has been deduced in the present work is summarized in Fig. 7. It is clear that at least one reason for the existence of several N-end-recognizing (E3) activities stems from the mechanistic requirement of recog-

TABLE II

Specific inhibition of X-βgal degradation by dipeptides

The effect of specific dipeptides on the degradation of *X*-βgal test proteins was measured as described in the legend to Fig. 6.

Residue *X* in *X*-βgal	Degradation of *X*-βgal (% of control) in the presence of a dipeptide		
	His-Ala	Trp-Ala	Ser-Ala
	2 mM	*2 mM*	*5 mM*
Arg	20	76	95
Lys	9	70	81
His	12	67	51
Asp	15	90	110
Glu	12	78	98
Cys	24	87	111
Asn	16	77	72
Gln	12	75	83
Phe	160	6	82
Leu	151	27	97
Trp	132	8	83
Tyr	127	11	74
Ala	85	64	16
Ser	84	92	26
Thr	80	71	17

nizing a chemically diverse set of primary destabilizing amino-terminal residues. The Type I E3 activity is specific for the set of destabilizing residues (Arg, Lys, His) whose common property is positive charge. The Type II E3 activity is specific for the set of bulky hydrophobic destabilizing residues, Phe, Leu, Trp, Tyr (and apparently also Ile in yeast; see below). The Type III E3 activity, present in reticulocytes but apparently absent from yeast (Table I and Fig. 7), is specific for the set of destabilizing residues (Ala, Ser, Thr) that share the properties of small size and lack of charge. It is likely, but remains to be proven, that these three N-end-recognizing activities correspond to more than one species of E3 protein.

A priori, the recognition of other chemically distinct sets of destabilizing amino-terminal residues such as Asp/Glu and Asn/Gln could be achieved by a similar E3-dependent mechanism, with the corresponding N-end-recognizing (E3) proteins being specific for either negatively charged amino-terminal Asp and Glu or their respective amides. However, the recognition of amino-terminal Asn/Gln and Asp/Glu (and also of Cys in reticulocytes) proceeds instead via a hierarchical system that conjugates these amino-terminal residues to a primary destabilizing residue (largely, but possibly not exclusively, Arg in both yeast and reticulocytes) (Fig. 7). This conjugation, carried out by a specific class of enzymes, aminoacyl-tRNA-protein transferases, proceeds directly with the secondary destabilizing residues, Asp and Glu (and also Cys in reticulocytes), but is preceded by a deamidation reaction in the case of the tertiary destabilizing residues, Asn and Gln (Figs. 7 and 8). Whether these seemingly avoidable enzymatic complexities serve specific regulatory functions remains to be determined.

Although most of the amino-terminal residues that confer short half-lives on *X*-βgal in yeast are destabilizing in reticulocyte extract as well, a closer examination of Table I shows that the relative degree of destabilization by a given residue is, in general, not the same in yeast and reticulocytes, even though the corresponding test protein *X*-βgal, is apparently the same in both cases. At the same time, arranging the primary destabilizing residues according to the type of E3 activity that recognizes them (Table III) reveals distinct areas of quantitative conservation between the yeast and reticulocyte N-end rules. In particular, the order of destabilizing "capacities" of the Type I primary destabilizing residues is the same in yeast and reticulocytes (Table III). This is also partly true for the Type II primary destabilizing residues, the conspicuous deviations being Leu and Ile, which are much less destabilizing in reticulocytes than in yeast (indeed, Ile is a "borderline" stabilizing residue in reticulocytes; see Fig. 7, Tables I and III, and discussion above). Both Leu and Ile have branched side chains, in contrast to the other hydrophobic destabilizing residues, Phe, Tyr, and Trp, which have aromatic side chains. Thus, the observed difference in the relative destabilizing capacities of Leu and Ile between yeast and reticulocytes may be due to a greater discrimination by the reticulocyte Type II E3 protein against branched hydrophobic residues, possibly because of the need to maximize the metabolic stability of proteins with amino-terminal Val, a smaller branched hydrophobic residue. Val is the strongest stabilizing residue in the reticulocyte N-end rule (Table I) and occurs in particular at the mature amino termini of both α- and β-chains of wild-type mammalian hemoglobin (Croft, 1980), the most abundant protein in erythrocytes and their precursors, reticulocytes.

Gln-βgal, whose amino-terminal residue is a tertiary destabilizing one, has a half-life of ~0.8 h in reticulocyte extract, which is slightly but reproducibly shorter than the half-life of Arg-βgal ($t_{½}$ of ~1.0 h; Table I). Arg, a primary destabilizing residue, is also the one that is conjugated to the amino terminus of Gln-βgal, after its preliminary conversion, via enzymatic deamidation, into Glu-βgal (Figs. 7 and 8). Thus, although it takes two additional enzymatic steps for Gln-βgal to acquire the amino-terminal Arg, its half-life is still shorter than that of Arg-βgal with the preexisting amino-terminal Arg. What might be the reason for this apparently paradoxical result? One consequence of Arg conjugation is the 1-residue increase in a distance between the first determinant of an *X*-βgal's degradation signal (the amino-terminal residue of *X*-

TABLE III

Comparison of destabilizing residues in yeast and reticulocytes

The half-life data of Table I were combined with the assignments of specific destabilizing residues to different classes (primary, secondary, tertiary; type I, II, III) that have been established in the present work (Figs. 5, 6, and Table II). The resulting arrangement reveals a greater similarity between quantitative aspects of the yeast and reticulocyte N-end rules than is apparent from the arrangement in Table I.

		Half-life of *X*-βgal	
		Yeast (*in vivo*)	Reticulocytes (*in vitro*)
Primary destabilizing residue *X*			
Type I	Arg	2 min	1.0 h
	Lys	3 min	1.3 h
	His	10 min	3.5 h
Type II	Phe	3 min	1.1 h
	Leu	3 min	5.5 h
	Trp	3 min	2.8 h
	Tyr	10 min	2.8 h
	Ile	30 min	20 h
Type III	Ala	>20 h	4.4 h
	Ser	>20 h	1.9 h
	Thr	>20 h	7.2 h
Secondary destabilizing residue *X*			
Asp		3 min	1.1 h
Glu		30 min	1.0 h
Cys		>20 h	1.2 h
Tertiary destabilizing residue *X*			
Asn		3 min	1.4 h
Gln		10 min	0.8 h

βgal) and the second determinant of the same degradation signal (a specific internal Lys residue of *X*-βgal) (Bachmair and Varshavsky, 1989). This increase is likely to augment the strength of the degradation signal (see Bachmair and Varshavsky (1989) for the definition and discussion of the amino-terminal degradation signal). It is also possible that the above effect is due to a modulating influence of the second residue in a target protein (Glu in Arg-Glu-His-Gly-βgal and His in Arg-His-Gly-βgal) (see Bachmair *et al.*, 1986; Bachmair and Varshavsky, 1989). Yet another possibility is that enzymes which act on secondary and tertiary destabilizing residues may influence kinetics of subsequent targeting steps, possibly through a physical association with "downstream" components of the degradative pathway (Fig. 8).

Are Long-lived and Short-lived X-βgal Proteins Degraded by a Common Pathway?—Long half-lives of the *X*-βgal proteins which bear *stabilizing* amino-terminal residues can be considered a "default" consequence of the absence of E3 proteins recognizing these residues. On the other hand, different stabilizing residues confer reproducibly different half-lives on the corresponding *X*-βgal proteins in reticulocyte extract (Table I). The same appears to be true of long-lived *X*-βgal proteins in yeast (Bachmair *et al.*, 1986).[3] The slow degradation of Met-, Gly-, and Ile-βgal in reticulocyte extracts is ATP-dependent, and apparently also ubiquitin-dependent (Figs. 3, 4, and data not shown). If long-lived and short-lived *X*-βgal proteins (Table I) are degraded by a common pathway, two distinct mechanisms could account for the presently available data: (i) a finite fidelity of the amino-terminal recognition of proteolytic substrates would result in rare events of binding of stabilizing amino-terminal residues by the N-end-recognizing (E3) proteins that normally recognize destabilizing amino-terminal residues; and (ii) a slow proteolytic "nibbling" at an *X*-βgal's amino terminus which at some point exposes a previously internal destabilizing residue at a new amino terminus, followed by rapid degradation of the thus modified βgal. Fine tuning of the rate of degradation in the latter case may be a function of the rate of slow amino-terminal cleavage that exposes a destabilizing residue, rather than a function of the residue's destabilizing capacity according to the N-end rule. It remains to be seen whether these mechanisms are relevant to the degradation of long-lived *X*-βgal proteins or, more generally, to the degradation of naturally occurring long-lived intracellular proteins.

The N-End Rule Pathway—The degradative pathway whose initial steps involve amino-terminal recognition of proteolytic substrates is called the *N-end rule pathway* (see Fig. 8 and its legend), to distinguish it from other proteolytic pathways and also from other ubiquitin-dependent processes (Jentsch *et al.*, 1987; Ball *et al.*, 1987; Goebl *et al.*, 1988; Siegelman and Weissman, 1988; Dunigan *et al.*, 1988; Pickart and Vella, 1988; Haas and Bright, 1988). Targeting by the N-end rule pathway requires the generation of a destabilizing residue at the amino terminus of a proteolytic substrate; the rules and mechanisms of these early events for the physiologically relevant substrates of the N-end rule pathway remain to be understood.

The consistent absence of 12 amino acids that are destabilizing according to the yeast N-end rule (Table I) from the mature amino termini of relatively long-lived, noncompartmentalized proteins (Bachmair *et al.*, 1986) is largely due to the substrate specificity of the enzyme methionine aminopeptidase. In both bacteria and eukaryotes, this enzyme (Tsunasawa *et al.*, 1985; Ben-Bassat *et al.*, 1987; Miller *et al.*, 1987; Huang *et al.*, 1987; Boissel *et al.*, 1988; Arfin and Bradshaw, 1988) cleaves off the amino-terminal Met residue if and only if it is *not* followed either by another Met residue or by any of the 12 residues that are destabilizing according to yeast N-end rule. Such cleavage specificity would be functionally relevant if the amino-terminal exposure of a (penultimate) destabilizing residue were to confer short half-lives on at least some of the otherwise long-lived proteins that normally retain their amino-terminal methionine. The inverse correspondence between the yeast N-end rule and the substrate requirements of the methionine aminopeptidase provides a partial functional explanation for the properties of this enzyme: a methionine-clipping aminopeptidase that is involved in processing of long-lived proteins would be expected *not* to expose a residue whose presence at the amino terminus might destabilize the substrate protein.

We suggest that analogous, still to be identified proteases may be responsible for the generation of amino termini bearing either primary, secondary, or tertiary destabilizing residues (Fig. 8) in specific proteins. An example of a noncompartmentalized short-lived protein whose amino-terminal processing yields a destabilizing amino-terminal residue is the phage λ cII protein, which is central to the lysis-lysogeny decision by λ, and in which the third residue, Arg, is found at the mature amino terminus (Ho *et al.*, 1986). (The existence of aminoacyl-tRNA-protein transferases in bacteria (Soffer, 1980), and a strong bias against destabilizing amino-terminal residues in long-lived, noncompartmentalized proteins from both bacteria and eukaryotes (Bachmair *et al.*, 1986) suggest that a N-end rule pathway exists in bacteria as well. Experimental testing of this conjecture is under way.) Specific endoproteolytic cleavages anywhere in a protein should also be able to generate destabilizing residues at the amino termini of cleavage products, and may be involved, for instance, in the targeting of damaged or otherwise abnormal proteins by the N-end rule pathway (Varshavsky *et al.*, 1988; Bachmair and Varshavsky, 1989).

Not shown explicitly in Fig. 8 are three important mechanistic features of the N-end rule pathway. One is the demonstrated multiplicity of the N-end-recognizing (E3) activities (see above and Figs. 6 and 7). The other is a bipartite nature of the amino-terminal degradation signal which has been shown to comprise two distinct determinants (Bachmair and Varshavsky, 1989). One determinant, discovered previously (Bachmair *et al.*, 1986) and studied in the present work (d_p in Fig. 8), is the amino-terminal residue of a proteolytic substrate. The second determinant is a specific, internal lysine residue of a proteolytic substrate (Bachmair and Varshavsky, 1989). Also not shown explicitly in Fig. 8 is the recently deciphered structure of a multiply ubiquitinated *X*-βgal protein in which an ordered chain of branched *ubiquitin-ubiquitin* conjugates was shown to be confined to a lysine residue that had been identified by molecular genetic analysis as the second determinant of the degradation signal (Chau *et al.*, 1989; Bachmair and Varshavsky, 1989).

Is the Exact Form of the N-end Rule a Function of the Cell's Physiological State?—Metabolic instability of the Ala-, Ser-, Thr-, and Cys-βgal proteins in reticulocytes and their stability in yeast is the major, qualitative difference between the yeast and reticulocyte N-end rules (Fig. 7 and Tables I and III). Since Cys is a secondary destabilizing residue in reticulocytes (Figs. 7 and 8), the metabolic stability of Cys-βgal in yeast (Tables I and III) may be due to the lack of arginylation of Cys-βgal in yeast, either because of a difference between substrate specificities of the yeast and reticulocyte arginine-tRNA-protein transferases or because Cys-βgal is amino terminally blocked in yeast cells but not in reticulocyte extract.

[3] A. Bachmair and A. Varshavsky, unpublished results.

Amino acid sequencing of reisolated X-βgal test proteins showed that the amino termini of Ala-βgal and Thr-βgal (which, in addition to Ser-βgal and Cys-βgal, are short-lived in reticulocytes but long-lived in yeast) are unblocked in both yeast cells and reticulocyte extract (Table I). The amino terminus of Ser-βgal was found to be blocked, presumably by acetylation, in yeast cells but not in reticulocyte extract (Table I). Thus, a "yeast-specific" amino-terminal modification (*e.g.* acetylation) of Ala- and Thr-βgal cannot be an explanation of the striking difference in relative metabolic stabilities of these test proteins between reticulocytes and yeast. These results, and our finding that Ala-, Ser-, and Thr-βgal are targeted for degradation by a distinct N-end-recognizing (E3) activity in reticulocytes (Figs. 6 and 7 and Table II) are consistent with the possibility that yeast cells lack the Ala-, Ser-, Thr-specific E3 activity.

The "Ala, Ser, Thr" divergence between the yeast and reticulocyte N-end rules (Fig. 7) appears particularly significant in view of the fact that Ala, Ser, and Thr are among the amino-terminal residues often present in long-lived, noncompartmentalized intracellular proteins (Bachmair *et al.*, 1986; Croft, 1980; Flinta *et al.*, 1986; Tsunasawa *et al.*, 1985). This, together with the presence in reticulocytes of a distinct N-end-recognizing (E3) activity specific for Ala, Ser, and Thr (Figs. 6 and 7 and Table II), leads us to suggest that the metabolic instability of Ala-, Ser-, and Thr-βgal in reticulocytes may be due to a reticulocyte-specific variant of the N-end rule. Selective degradation of undamaged, previously long-lived proteins is unusually extensive during the conversion of reticulocytes into erythrocytes but apparently occurs (and may be essential) in most if not all other cell lineages as well. This putative differentiation-dependent protein degradation via the N-end rule pathway could be achieved through the regulation of either specific N-end-recognizing (E3) proteins or enzymes that act upon primary, secondary, and tertiary destabilizing residues (Fig. 8). If the exact form of the N-end rule is indeed a function of the cell's physiological state, it could provide a mechanism for selective destruction of preexisting, otherwise long-lived and undamaged proteins during cell differentiation, cell cycle progression, and other changes in the cell's state. Experimental testing of this possibility is under way.

Acknowledgments—We thank members of this laboratory, especially Bonnie Bartel, Daniel Finley, and Mark Hochstrasser, for their comments on the manuscript, and Barbara Doran for secretarial assistance.

REFERENCES

Arfin, S. M., and Bradshaw, R. A. (1988) *Biochemistry* **27,** 7979–7984
Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., and Struhl, K. (eds) (1987) *Current Protocols in Molecular Biology*, John Wiley & Sons, New York
Bachmair, A., Finley, D., and Varshavsky, A. (1986) *Science* **234,** 179–186
Bachmair, A., and Varshavsky, A. (1989) *Cell* **56,** 1019–1032
Bahl, H., Echols, H., Strauss, D. B., Court, D., Crowl, R., and Georgopoulos, K. (1987) *Genes Dev.* **1,** 57–64
Banuett, F., Hoyt, M. A., McFarlane, L., Echols, H., and Herskowitz, I. (1986) *J. Mol. Biol.* **187,** 213–224
Ben-Bassat, A., Bauer, K., Chang, S.-Y., Myambo, K., Boosman, A., and Chang, S. (1987) *J. Bacteriol.* **169,** 751–757
Boissel, T. J., Kasper, T. J., and Bunn, H. F. (1988) *J. Biol. Chem.* **263,** 8443–8449
Bond, J. S., and Butler, P. E. (1987) *Annu. Rev. Biochem.* **56,** 333–364
Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D., Gonda, D. K., and Varshavsky, A. (1989) *Science* **243,** 1576–1583
Cheng, H. H., and Echols, H. (1987) *J. Mol. Biol.* **196,** 737–740
Ciechanover, A., Finley, D., and Varshavsky, A. (1984) *Cell* **37,** 57–66
Croft, L. R. (1980) *Handbook of Protein Sequence Analysis*, John Wiley & Sons, New York
Deutch, C. (1984) *Methods Enzymol.* **106,** 198–205
Dice, J. F. (1987) *FASEB J.* **1,** 349–357
Dunigan, D. D., Dietzgen, R. G., Schoelz, J. E., and Zaitlin, M. (1988) *Virology* **165,** 310–312
Etlinger, J. D., and Goldberg, A. L. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 54–58
Ferber, S., and Ciechanover, A. (1986) *J. Biol. Chem.* **261,** 3128–3134
Ferber, S., and Ciechanover, A. (1987) *Nature* **326,** 808–811
Finley, D., Ciechanover, A., and Varshavsky, A. (1984) *Cell* **37,** 43–55
Finley, D., and Varshavsky, A. (1985) *Trends Biochem. Sci.* **10,** 343–346
Finley, D., Özkaynak, E., Jentsch, S., McGrath, J. P., Bartel, B., Pazin, M., Snapka, R. M., and Varshavsky, A. (1988) in *Ubiquitin* (Rechsteiner, M., ed) pp. 39–75, Plenum Publishing Co., New York
Finley, D., Özkaynak, E., and Varshavsky, A. (1987) *Cell* **48,** 1035–1046
Finley, D., Bartel, B., and Varshavsky, A. (1989) *Nature* **338,** 394–401
Flinta, C., Persson, B., Jornvall, H., and von Heijne, G. (1986) *Eur. J. Biochem.* **154,** 193–196
Goebl, M., Yochem, J., Jentsch, S., McGrath, J. P., Varshavsky, A., and Byers, B. (1988) *Science* **241,** 1331–1335
Gottesman, S., Gottesman, M., Shaw, J. E., and Pearson, M. L. (1981) *Cell* **24,** 225–233
Haas, A. L., and Bright, P. M. (1988) *J. Biol. Chem.* **263,** 13258–13267
Hershko, A., Ciechanover, A., Heller, H., Haas, A. L., and Rose, I. A. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 1783–1786
Hershko, A., and Ciechanover, A. (1986) *Prog. Nucleic Acids Res. Mol. Biol.* **33,** 19–56
Hershko, A. (1988) *J. Biol. Chem.* **263,** 15237–15240
Ho, Y. S., Wulff, D., and Rosenberg, M. (1986) in *Regulation of Gene Expression* (Booth, I., and Higgins, C., eds) pp. 79–103, Cambridge University Press, London
Huang, A., Elliot, R. C., Liu, P. S., Koduri, R. K., Weickman, J. L., Lee, J. H., Blair, L. C., Ghosh-Dastidar, P., Bradshaw, R. A., Bryan, K. M., Einarson, B., Kendall, R. L., Kolacz, K. H., and Saito, K. (1987) *Biochemistry* **26,** 8242–8246
Ingoglia, N. A., Giuditta, A., Zanakis, M. F., Babigian, A., Tasaki, I., Chakraborty, G., and Sturman, J. A. (1983) *J. Neurosci.* **3,** 2463–2473
Jentsch, S., McGrath, J. P., and Varshavsky, A. (1987) *Nature* **329,** 131–134
Kaji, A., and Novelli, G. D. (1965) *J. Biol. Chem.* **240,** 1185–1197
Kehry, M. R., Bond, M. W., Hunkapiller, M. W., and Dahlquist, F. W. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 3599–3603
Laemmli, U. K. (1970) *Nature* **227,** 680–685
Maicas, E., Pluthero, F. G., and Friesen, J. D. (1988) *Mol. Cell. Biol.* **8,** 169–175
Matsudaira, P. (1987) *J. Biol. Chem.* **262,** 10035–10038
Miller, C. G., Strauch, K. L., Kukral, A. M., Miller, J. L., Wingfield, P. T., Mazzei, G. J., Werlen, R., Graber, P., and Movva, N. R. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 2718–2722
Nagai, K., and Thorgensen, H. (1987) *Methods Enzymol.* **153,** 461–481
Nasmyth, K. (1983) *Nature* **302,** 670–676
Özkaynak, E., Finley, D., Solomon, M. J., and Varshavsky, A. (1987) *EMBO J.* **6,** 1429–1439
Pickart, C. M., and Vella, A. T. (1988) *J. Biol. Chem.* **263,** 15076–15082
Reiss, Y., Kaim, D., and Hershko, A. (1988) *J. Biol. Chem.* **263,** 2693–2698
Shanklin, J., Jabben, M., and Vierstra, R. D. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 359–363
Shyne-Athwal, S., Riccio, R. V., Chakraborty, G., and Ingoglia, N. A. (1986) *Science* **231,** 603–605
Siegelman, M., and Weissman, I. L. (1988) in *Ubiquitin* (Rechsteiner, M., ed) pp. 239–269, Plenum Publishing Co., New York
Smith, M. (1985) *Annu. Rev. Genet.* **19,** 423–462
Soffer, R. L. (1980) in *Transfer RNA: Biological Aspects* (Söll, D., Abelson, J., and Schimmel, P. R., eds) pp. 493–505, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Spindler, K., and Berk, A. J. (1984) *J. Virol.* **52,** 706–710
Straus, D. B., Walter, W. A., and Gross, C. A. (1987) *Nature* **329,** 348–351
Terwilliger, T. C., and Koshland, D. E., Jr. (1984) *J. Biol. Chem.* **259,** 7719–7725
Tsay, Y. F., Thompson, J. R., Rotenberg, M. O., Larkin, J. C., and Woolford, J. L., Jr. (1988) *Genes Dev.* **2,** 664–676
Tsunasawa, S., Steward, J. W., and Sherman, F. (1985) *J. Biol. Chem.* **260,** 5382–5391
Ullmann, A. (1984) *Gene (Amst.)* **29,** 27–31
Varshavsky, A., Bachmair, A., Finley, D., Gonda, D. K., and Wünning, I. (1988) in *Ubiquitin* (Rechsteiner, M., ed) pp. 287–324, Plenum Publishing Co., New York